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T-cell-based immunity counteracts the potential toxicity of glutamate in the central nervous system

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Abstract

Injuries to the central nervous system (CNS) evoke self-destructive processes, which eventually lead to a much greater loss of tissue than that caused by the trauma itself. The agents of self-destruction include physiological compounds, such as glutamate, which are essential for the proper functioning of the CNS, but become cytotoxic when their normal concentrations are exceeded. The CNS is equipped with buffering mechanisms that are specific for each compound. Here we show, using Balb/c mice (a strain resistant to induction of experimental autoimmune encephalomyelitis), that after intravitreal injection of any concentration of glutamate (a neurotransmitter that becomes toxic when in excess) or ammonium–ferrous sulfate hexahydrate (which increases the formation of toxic oxygen species), the loss of retinal ganglion cells in mice devoid of mature T cells (nude mice) is significantly greater than in matched wild-type controls. We further show that this outcome can be partially reversed by supplying the T cell-defective mice with splenocytes derived from the wild-type mice. The results suggest that potentially toxic physiological compounds, when present in excessive amounts, can recruit and activate a T-cell-dependent self-protective immune mechanism. This may represent a prototype mechanism for the physiological regulation of potentially destructive CNS events by T-cell-mediated immune activity, when the local buffering mechanisms cannot adequately cope with them.

Keywords: Beneficial autoimmunity; Neuroprotection; CNS injury; Glutamate toxicity; Oxidative stress; CNS degeneration

1. Introduction

Conditions of acute and chronic stress in the CNS result in increased concentrations of glutamate and free radicals, both considered to be primarily responsible for propagation of degeneration after acute insults, and are also associated with chronic degeneration (Coyle and Puttfarcken, 1993; Ikonomidou and Turski, 1995). Excitotoxicity, caused by excessive amounts of glutamate, appears to be acutely involved in status epilepticus (Fountain, 2000), as well as after cerebral ischemia (Dirnagl et al., 1999; Lipton, 1999) and traumatic brain or axonal injuries (Alessandri and Bullock, 1998). It may also contribute to the chronic neurodegeneration seen in amyotrophic lateral sclerosis, Huntington's chorea, glaucoma, and other CNS disorders (Kieburtz, 1999; Meldrum, 2000; Shaw and Ince, 1997). Accumulation of glutamate in these pathological conditions can result from excessive glutamate release and/or its insufficient removal. An example of a neurodegenerative disease associated with a local increase in glutamate is the optic nerve neuropathy seen in glaucoma patients (Dreyer, 1998; Vorwerk et al., 1999). The presence of abnormally high amounts of glutamate in the eyes of these patients is thought to be responsible, at least in part, for the continual death of RGCs despite the use of medications which reduce primary risk factors such as intraocular pressure (Dreyer et al., 1996). Oxidative stress is similarly associated with degenerative disorders of the CNS.

The CNS is equipped with specific mechanisms for buffering excessive quantities of such compounds (Kanai et al., 1993; Sims and Robinson, 1999; Skaper et al., 1999). These mechanisms may be viewed as local defense mechanisms against self-compounds which, though essential for normal CNS functioning, need to be rigorously regulated to avoid any dangerous increase in their concentrations. Apparently, however, these constitutive defense mechanism are effective only to a certain degree, beyond that limit they can no longer cope with the stress, resulting in neuronal death (Obrenovitch, 1999; Obrenovitch et al., 2000).

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We recently observed that mechanical injuries to the CNS evoke mechanisms of self-protection mediated by T cells specific to CNS myelin proteins (Yoles et al., 2001a). Since the damage inflicted by these injuries is often exacerbated by physiological compounds (such as glutamate) acting as mediators of toxicity (Yoles and Schwartz, 1998), we were interested in finding out whether the protective T-cell-mediated mechanisms might be evoked by these same physiological compounds. This question was addressed in the present study, using an in vivo model of neurotoxicity induced by glutamate or free radicals in the visual systems of nude mice, in which the ability to develop mature T cells was defective, and in their matched wild-type controls. We show here that both glutamate and free radicals, at any concentration, cause a larger neuronal loss in retinas of the transgenic mice without normal T-cell development, than in wild-type control retinas. We further show that this result can be reversed, in part, by supplying the T cell-defective mice with splenocytes derived from the wild-type mice. This finding suggests that the excess neuronal loss in the T-cell deficient mice relative to wild-type, is due, at least in part to defective immune cells.

2. Materials and methods

2.1. Animals

Animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee. Wild-type or nude mice of the Balb/c strain, aged 8–13 weeks, were supplied by the Animal Breeding Center of The Weizmann Institute of Science and housed in light-and temperature-controlled rooms. Prior to all experiments, the mice were anesthetized by intraperitoneal administration of ketamine 80 mg/kg and xylazine 16 mg/kg.

2.2. Injections of glutamate, ammonium–ferrous sulfate hexahydrate, and NMDA

Under binocular microscope, the right eye of the anesthetized mouse was punctured with a 27-gauge needle in the upper part of the sclera and a 10-μl Hamilton syringe with a 30-gauge needle was inserted as far as the vitreal body. Mice were injected with a total volume of 1 μl of L-glutamate (Sigma), or 1 μl of ammonium–ferrous sulfate hexahydrate (Merck, Darmstadt, Germany), or 1 μl of *N*-methyl-D-aspartate (NMDA; 75 nmol; RBI, Boston, MA) dissolved in saline.

2.3. Application of stereotactic dye

The mice were anesthetized and their skull exposed. The bregma was identified and marked. A hole was drilled above the superior colliculus of each hemisphere (2.92 mm posterior to the bregma and 0.5 mm lateral to the midline). Using a stereotactic measuring device and a Hamilton injector, the mice were injected with FluoroGold (5% in saline, Fluorochrome, Denver, CO; 1 μl per 2 min) at one point in the superior colliculus of each hemisphere, at a depth of 1.8 mm from the surface of the brain. After completion of the injection the skin was sutured. Glutamate injection to the eye causes cell death and not blockage of axonal transport and therefore, retrograde uptake of the dye by the retinal ganglion cells provides a marker for the viable cells.

2.4. Assessment of retinal ganglion cell survival

Mice were given a lethal dose of pentobarbitone (170 mg/kg). Their eyes were enucleated and the retinas were detached and prepared as flattened whole mounts in paraformaldehyde (4% in PBS). Labeled cells from four to six selected fields of identical size (0.078 mm^2), which were located at approximately the same distance from the optic disk, were counted under the fluorescence microscope (magnification × 800) by observers blinded to the treatments received. The average number of RGCs per field in each retina was calculated.

2.5. Transfer of splenocytes into nude mice

Spleens were removed aseptically from wild-type Balb/c mice and suspended in Hank's balanced salt solution (HBSS, Sigma) and 5% fetal calf serum (FCS). The splenic capsule was removed and debris from the cell suspension was allowed to settle for 5 min at room temperature. The cells were then centrifuged at 200–250 × *g* for 10 min at 4 °C. Red blood cells were lysed with 0.8% ammonium chloride and 0.1% potassium hydrogen carbonate dissolved in double-distilled water for 2–4 min at room temperature. The remaining cells were washed in RPMI (containing 10% FCS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μM 2-mercaptoethanol), and centrifuged at 200–250 × *g* for 15 min at 4 °C. The pellet was washed with RPMI and centrifuged at 200–250 × *g* for 10 min at 4 °C. Splenocytes were resuspended in HBSS (7×10^7 cells/ml), and 0.5 ml of the suspension was injected intravenously into the lateral tail veins of nude mice on the day of glutamate injection.

3. Results

3.1. Glutamate induces a T-cell-dependent protection

To determine whether glutamate in excessive amounts triggers an immune system-related protective mechanism, we compared the neurotoxic effects of intravitreal injections of increasing amounts of glutamate, unilaterally into

the eyes of wild-type and nude Balb/c mice. The choice of Balb/c mice was based on our previous studies, demonstrating that the survival rate in Balb/c mice following intravitreal injection of glutamate, is higher than in C57bl/6 mice (Kipnis et al., 2001). Every experiment included labeling of retinas in a control group, which was either not injected, or was injected only with saline. Intravitreal glutamate injection causes death of RGCs (Schori et al., 2001; Yoles et al., 2001a). Seven days after injection, retinas from both types were excised and the numbers of surviving (retrogradely labeled) RGCs counted. Intravitreal injection of 40 nmol of glutamate (an amount which did not cause RGC death in the wild type) had a significant neurotoxic effect in the retinas of mice devoid of mature T cells (nude mice) (Fig. 1). However, in the retinas of non-injected eyes the numbers of RGCs per mm^2 counted in the wild-type and the nude mice were similar (3097 ± 44, $n = 6$, and 2937 ± 131, $n = 8$, respectively). These findings suggest that glutamate is potentially toxic even at low concentrations, but that in wild-type (normal) mice the toxicity is not expressed, probably because it is counteracted by a glutamate-evoked protective mechanism associated with T cells. Presumably, the effect of the toxicity is apparent only when glutamate concentration elevates too high for this protective mechanism to cope with it. We wanted to determine whether the heightened vulnerability to glutamate toxicity in RGCs of T-cell-deficient mice, relative to the wild-type, is indeed related to the deficient immune system and not to any local CNS defective mechanism yet unknown, associated with the defect in the Thymus. We therefore transferred splenocytes from wild-type mice to nude mice immediately following glutamate injection to the latter. Following this procedure, neuronal loss in the nude mice were significantly reduced (Fig. 2).

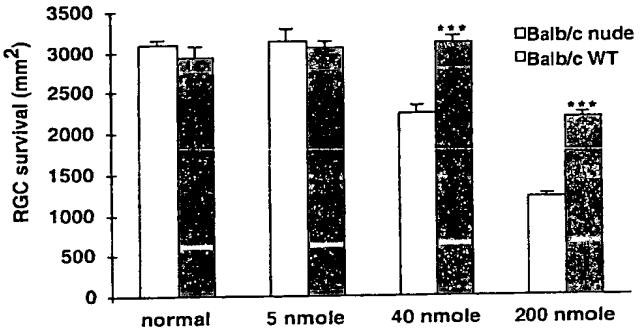

Fig. 1. T-cell-dependent neuroprotection evoked by glutamate. Wild-type and nude mice of the Balb/c strain were injected intravitreally with different dosages of glutamate, and 7 days later their RGCs were counted as described in Materials and methods. There were no significant differences in the numbers of RGCs between wild-type mice ($n = 4$) and nude mice ($n = 4$) injected with 5 nmol of glutamate ($p < 0.68$, t-test). However, following injection with 40 or 200 nmol of glutamate, the differences between wild-type and nude mice ($n = 6$–13) were very significant ($p < 10^{-5}$, t-test).

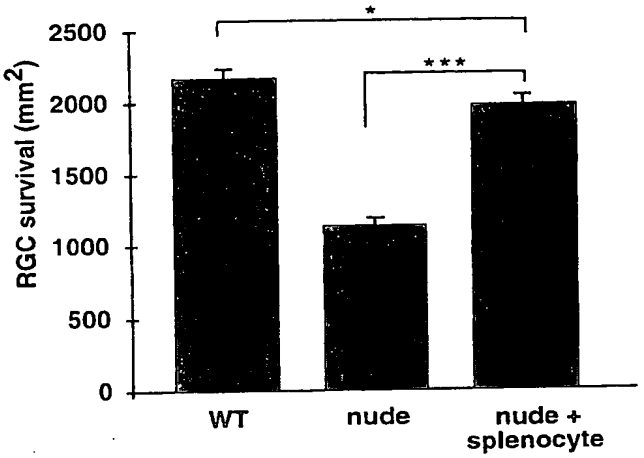

Fig. 2. Splenocytes from wild type can compensate for glutamate-induced neuronal loss in T-cell defective mice. Wild-type and nude Balb/c mice were injected intravitreally with 200 nmol glutamate. The nude mice received, in addition, intravenous injections of $3-4\times10^7$ splenocytes from naive Balb/c wild-type mice (see Materials and methods). After 7 days, RGCs were counted as described. Significant reduction in the loss of RGCs was observed in glutamate-injected nude mice that also received splenocytes ($n = 10$) relative to nude mice that were injected with glutamate only ($n = 6$; $p < 0.0019$, t-test). It should be noted, however, that reconstitution with splenocytes under our experimental conditions was not complete, as the numbers of surviving RGCs in the splenocyte-injected nude mice ($n = 6$) was still lower than in the wild-type mice ($n = 13$; $p < 0.04$, t-test).

3.2. Free radicals evoke a T-cell-dependent protection

To determine whether the observed T-cell-dependent immune neuroprotection is recruited only in the case of glutamate toxicity, we used the same experimental paradigm to examine immune neuroprotection following exposure of RGCs to free radicals, another physiological risk factor. Ferrous ions are known to increase the formation of oxygen species in the tissue. Balb/c mice were injected intravitreally with various amounts of ammonium–ferrous sulfate hexahydrate, and their surviving RGCs were counted 1 week later. Every experiment included labeling of retinas, in a non-treated control group. Increasing Fe^{2+} concentrations evoked progressively more severe toxicity, measured in terms of RGC loss (Fig. 3A). In subsequent experiments we used a concentration of 10 nmol of ferrous ions, which in the wild-type mice caused loss of approximately 13% of their RGCs. To determine whether the stress associated with oxygen species (free radicals) evokes a beneficial T-cell-dependent response, we compared the extent of RGC loss in nude Balb/c mice to that in the wild-type after intravitreal injection of 10 nmol of ammonium–ferrous sulfate hexahydrate. As with glutamate injections, the numbers of surviving RGCs per mm^2 were significantly lower in the nude mice (488 ± 315, $n = 7$) than in the wild-type controls (2219 ± 84, $n = 10$) (Fig. 3B).

3.3. NMDA does not evoke a T-cell-dependent protection

Both glutamate and free radicals are physiological compounds that normally accumulate during neuronal activity. Intensive activity is likely to cause transient local accumulation of these agents in potentially toxic concentrations. The existence of a self-defense mechanism against such toxicity is therefore essential. We wanted to further investigate whether a neuroprotective autoimmune response can be triggered and effective in cases of toxicity caused by non-physiological neurotoxic agents. We subjected both wild-type and nude Balb/c mice to intravitreal injections of the glutamate agonist NMDA, an agent which was recently shown in our laboratory to cause death of RGC via a different mechanism than that mediated by glutamate (Yoles et al., 2001). Every experiment included labeling of retinas, in a non-treated control group. No differences in RGC survival rates were found between wild-type and nude mice following NMDA insult (wild-type, 1066 ± 69, $n = 12$; nude mice, 1004 ± 30, $n = 4$; Fig. 4). These findings may suggest that the immune system is not alerted by non-physiological compounds, or that its activity is not sufficient to counteract the speedy damage caused by non-physiological compounds (Yoles and Schwartz, 2001).

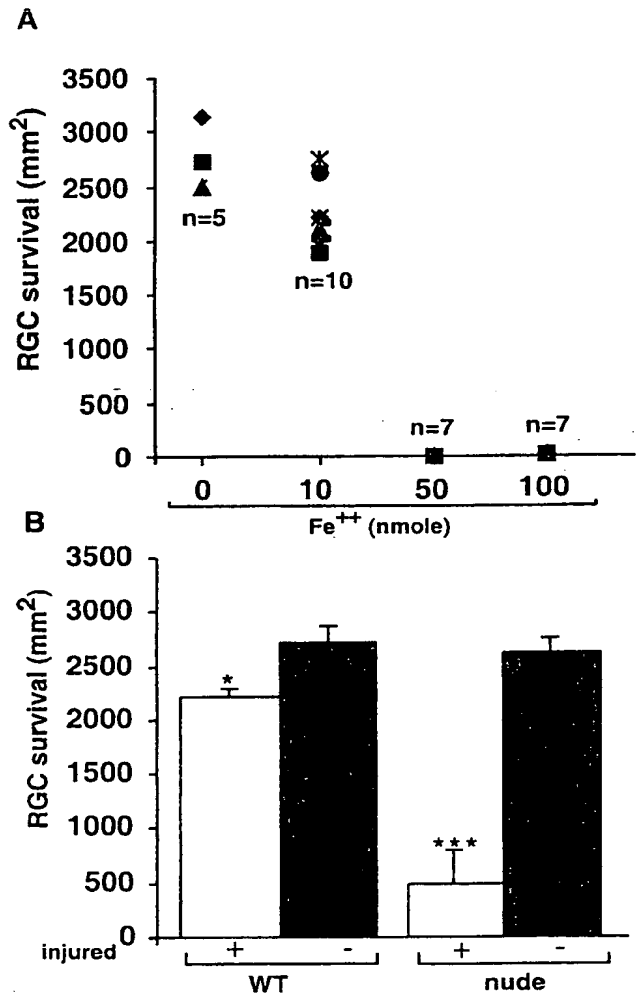

Fig. 3. T-cell-dependent neuroprotection evoked by oxidative stress. (A) Dose-dependent effect of intravitreal injection of ammonium–ferrous sulfate hexahydrate on RGC survival. Wild-type Balb/c mice were injected intravitreally with different dosages of ammonium–ferrous sulfate hexahydrate, and 7 days later their RGCs were counted as described in Materials and methods. (B) Protective autoimmune response evoked by ammonium–ferrous sulfate hexahydrate. Wild-type and nude Balb/c mice were injected intravitreally with 10 nmol ammonium–ferrous sulfate hexahydrate, and 7 days later their RGCs were counted as described in Materials and methods. The numbers of RGCs differed significantly between injected ($n = 10$) and non-injected ($n = 6$) wild-type mice ($p < 0.01$, t-test), and very significantly between injected ($n = 7$) and non-injected ($n = 6$) nude mice ($p < 0.0006$, t-test).

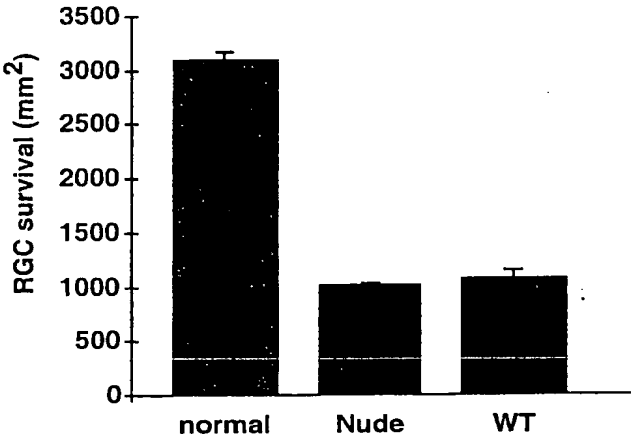

Fig. 4. NMDA toxicity in wild-type and nude Balb/c mice. Mice were injected intravitreally with 75 nmol NMDA, and 7 days later their RGCs were counted as described in Materials and methods. There were no significant differences in the numbers of RGCs between wild-type mice ($n = 12$) and nude mice ($n = 4$; $p < 0.66$, t-test).

4. Discussion

The results of this study show that abnormally high concentrations of physiological agents, known to mediate cytotoxicity, evoke a T-cell-dependent protective mechanism that helps to reduce their potential toxicity. This mechanism might represent a prototype mechanism for the regulation of endogenous compounds that have essential physiological roles on the one hand, but are potentially detrimental on the other.

Studies in our laboratory have shown that CNS axonal injury (optic nerve or spinal cord contusion) evokes a neuroprotective autoimmune response (Yoles et al., 2001b). We attributed the triggering of this beneficial autoimmunity to stress signals transmitted from the damaged myelinated axons (Schwartz, 2000; Schwartz and Cohen, 2000; Schwartz et al., 1999). This explanation was supported by the finding that active or passive T-cell-based immunity

directed against CNS-myelin-associated antigens reduce the neuronal loss associated with CNS damage (Fisher et al., 2001; Hauben et al., 2000; Moalem et al., 1999).

In the present study, using a paradigm of biochemical insult, we show that T-cell-mediated protection is involved in reducing neuronal loss, imposed by the potential toxic levels of physiological compounds. The activation of such protective mechanism occurs even before any damage is noticeable (no death was noticed at 40 nmol in the wild type). This finding might suggest that the signaling to the immune system comes from the potentially toxic compounds themselves, or alternatively, that minimal changes induced by the potentially toxic compounds are enough to alert the immune system. These interpretations appear to be in line with the observed lack of difference in RGC survival rate between nude mice and their wild-type controls after intravitreal injection of the non-physiological, ionotropic glutamate receptor agonist, NMDA.

Taken together, our findings appear to ascribe an additional function, hitherto unrecognized, to the immune system. The T-cell-mediated immune response has traditionally been viewed as a defensive mechanism against non-self that evolved to provide a versatile backup when the innate (mainly macrophage-related) response to invasion by pathogens is unequal to the task. In this respect a number of activities have been attributed to it, and any malfunctioning of this T-cell-mediated response was assumed to be harmful for the individual. Our studies provide evidence that stressful conditions, caused by a pathological increase in potentially toxic self compounds (e.g. glutamate and reactive oxygen species), might exceed the constitutive buffering capacity of the innate neural system and thus activate the T-cell-mediated immune response against self to provide protective T-cell-mediated activity.

The discovery that T-cell-mediated immune response, possibly directed against self compounds, apparently acts as a second line of defense against toxic conditions associated with physiological compounds, would appear to demand a reassessment of the role of the protective immunity in general and autoimmunity in particular (Schwartz and Kipnis, 2001).

At this stage, it is not clear what is the antigen specificity associated with the T-cell-mediated protection from glutamate toxicity and oxidative stress. It is possible that CNS insults of different types might essentially evoke similar protective physiological immune responses, yet the antigenic specificities of these responses may differ. Thus, for example, while immunization with myelin-associated antigens is beneficial in the context of axonal injury, it is not helpful against glutamate toxicity. Mice immunized with myelin oligodendrocyte glycoprotein (MOG) emulsified in complete Freund's adjuvant (CFA) obtain neuroprotective benefit only in the case of mechanical injury to the axons, not when the insult is biochemical (Fisher et al., 2001; Schori et al., 2001). On the other hand, neuroprotection against glutamate insult is obtained by immunizing the mice with Copolymer 1 (Cop-1), a synthetic peptide shown to cross-react with T cells directed against myelin-associated antigens (Kipnis et al., 2000; Schori et al., 2001). The fact that myelin-antigens provide effective protective immunization only in the case of axonal injury, and not in the case of glutamate toxicity, while Cop-1 immunization is effective in both cases, argues in favor of Cop-1 activating regulatory T-cells that are essential, though not sufficient, for the protective T-cell-based immunity (Schwartz and Kipnis, 2001).

It has yet to be determined how the evoked T-cell-dependent protective response ameliorates the potential damage and whether it acts locally or systemically. Works in our group have shown that the extent of the inflammatory response is not indicative to the protective or destructive effect on injured neuronal cells. We have yet to define the type of immune-cells which are involved in neuroprotection, their antigen specificity and their phenotype. It is possible that the response to self compounds that exceed physiological levels is a component of the more global dialog between the immune and the nervous systems, which operates to preserve the homeostasis of the individual under normal conditions and not only under stress. This would mean that an autoimmune response can be evoked by any deviation from the normal. Our results may broaden the indications for therapy of stroke, ischemia, trauma, or other chronic degenerative conditions by T-cell-based vaccination to maintain the balance against toxicity induced under extreme conditions of glutamate upsurge.

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